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Intravenous fat induces changes in PUFA and their bioactive metabolites: Comparison between Japanese and Australian preterm infants

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ABSTRACT

Objective: Oxylipins are biologically active signaling molecules that initiate and resolve inflammation; they are synthesized by oxidation of polyunsaturated fatty acids (PUFAs) and reflect PUFA intake and status. The PUFA intake in preterm infants differs between countries because of the type of lipid emulsions used and the PUFA content of breast milk. We compared total blood PUFA, free PUFA and their oxylipin levels in dried whole blood samples from preterm infants born in Australia and Japan.

Methods: We enrolled 30 and 14 preterm infants born less than 31 weeks' gestation, from Adelaide and Japan respectively. Blood samples were obtained from cord blood, and on postnatal days 4, 7, 14 and 28. Total PUFAs were measured using gas chromatography, while free fatty acids and oxylipins were screened using ultra high-performance liquid chromatography mass spectroscopy.

Results: Differences in the levels of blood PUFA between the centres were found which were in line with the timing and type of lipid emulsion administration. Significant differences in longitudinal levels were seen more often in free PUFA and their oxylipins than in total blood PUFA. This was particularly true for AA and DHA. In contrast, differences in the levels could be seen in total blood EPA, as well as in free EPA and its oxylipins. Further, levels of many free PUFA and their oxylipins were higher in Japanese infants than in Australian infants.

Conclusion: Differences in total and free fatty acids and unesterified oxylipins, were observed during the first weeks of life and between preterm infants born in Australia and Japan, which were likely a reflection of the type of lipid emulsion and timing of administration. The clinical significance of these changes remains to be explored.

1. Introduction

Very preterm infants (<32 weeks' gestation) frequently receive parenteral nutrition which is progressively replaced by an increasing enteral intake (breast milk and/or formula) as they transition to full enteral feeding. The fats of both sources of nutrition contain a range of fatty acids including the essential polyunsaturated fatty acids (PUFA), including the omega-6 linoleic acid (LA) and the omega-3 alpha linolenic acid (ALA), as well as their long chain PUFA metabolites arachidonic acid (AA - an omega-6 derivative), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both omega-3 derivatives.

The majority of fats in the circulation, whether derived from tissue sources, the diet or administered intravenously, exist esterified into triglycerides, phospholipids or cholesteryl esters. In response to various

Abbreviations: AA, arachidonic acid; ALA, linolenic acid; CI, confidence intervals; DBS, dried blood spot; DHA, docosahexaenoic acid; DiHOME, dihydroxy-octadecenoic acid; EET, epoxy-eicosatrienoic acid; EPA, eicosapentaenoic acid; EpDPA, epoxy docosapentaenoic acid; EpHOME, epoxy-hydroxy-octadecenoic acid; FAME, fatty acid methyl esters; FFA, free fatty acid; FID, flame ionisation detector; GMR, ratio of geometric means; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; IQR, inter-quartile range; JSH, Juntendo Shizuoka Hospital, Shizuoka Prefecture, Japan; LA, linoleic acid; LTB4, leukotriene B4; LX, lipoxin; oxo-ODE, oxo-octadecadienoic acid; PUFA, polyunsaturated fatty acids; WCH, Women's and Children's Hospital, Adelaide, Australia

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medical and physiological stressors, diverse lipases cause the release of free fatty acids (FFA) from these stores. In the case of the PUFA, their FFA can subsequently be oxidized via enzymatic processes (lipoxygenase, cyclooxygenase and cytochrome P450) or via non-enzymatic free radical oxidation to produce a variety of biologically active molecules known as oxylipins [1]. These oxylipins play important roles with respect to inflammation and its resolution, platelet aggregation, vasodilatation and tissue repair processes [2-4].

Studies in adults have reported that oxylipins derived from LA may be related to inflammatory disorders [5-9]. The subsequent demonstration that a reduction in dietary LA resulted in a reduction in levels of these LA-derived oxylipins, and an improvement in clinical signs, supports a role of these oxylipins in the pathogenesis of some disease states, for e.g. atherosclerosis [10]. Conversely, in a neonatal animal model of hyperoxia-induced lung disease, DHA and AA derived oxylipins markedly reduced lung injury, suggesting there may be a therapeutic role for selected oxylipins in ameliorating neonatal lung disease [11]. High levels of anti-inflammatory and pro-resolving oxylipins were recently detected in human milk, suggesting a role in gut function and immunity in the newborn [12].

PUFA are crucial nutrients in preterm infants and are important for normal development of the central nervous system, with DHA playing important roles in cognitive and visual development [13]. The PUFA intake in very preterm infants may differ between countries because of the different types of intravenous lipid emulsions administered and intrinsic differences in the PUFA profiles of breast milk. In Australia, very preterm infants typically receive SMOFlipid® (Fresenius Kabi, Uppsala, Sweden) made from a combination of soy oil, medium-chain triglycerides, olive oil and fish oil [14]. By contrast, very preterm infants born in Japan typically receive a soy oil-based lipid emulsion (Intralipos®, Ohtsuka Pharmaceutical, Tokyo, Japan). Japanese infants may therefore receive higher intakes of LA during the parenteral phase of nutrition, while Australian infants receive higher amounts of EPA and DHA. Furthermore, the PUFA profiles of breast milk differ between countries due to differences in the maternal diet [15, 16]. However, no published studies have assessed the impact of differences in the combined parenteral/enteral intake of PUFA on FFA and oxylipin levels in very preterm infants. Given the known biological activity of these compounds we aimed to determine whole blood PUFA, omega-6 and omega-3 derived FFA and oxylipin levels in very preterm infants born in Australia and Japan, and to explore the relationship between fat intake, FFA and oxylipin levels.

2. Methods

2.1. Study setting

This study was conducted at the Women's and Children's Hospital (WCH) (Adelaide, Australia) and the Juntendo Shizuoka Hospital (JSH) (Shizuoka Prefecture, Japan). The study protocols were approved by the Human Research Ethics Committee of each institute (WCH: HREC/17/WCHN/16, JSH: 29.179).

Preterm infants born less than 31 weeks' gestation were eligible for inclusion. Infants with suspected metabolic disease, major congenital or chromosomal abnormality were excluded. A cord blood sample was collected at birth in all eligible infants. Investigators then approached parents within 4 days of birth, and written informed consent was obtained; cord blood samples of infants whose families did not consent to participate were discarded.

2.2. Sample collection

Blood samples (two drops of whole blood) were collected at birth (cord blood), and on postnatal days 4, 7, 14 and 28 (via heel prick) and spotted on PUFAcoat® cards [17]. Breast milk samples were collected on postnatal days 7, 14 and 28. and spotted on to PUFAcoat® cards. Infants receiving continuous or intermittent tube feeds, or bottle feeds had two drops of breast milk taken directly from the tubing or bottle. For those breast feeding at the time of sample collection women were asked to express two drops of milk directly onto the PUFAcoat® card. All samples were air dried on a drying rack at room temperature for at least 3 h and stored at −20 °C until analysis.

2.3. Laboratory analysis

All fatty acid analyses were undertaken in the fatty acid laboratory at the School of Agriculture Food and Wine, The University of Adelaide. Thirty-two oxylipins (**Supplementary Table 1)** and 5 total and FFA (LA, AA, ALA, EPA and DHA) were screened in whole blood and breast milk.

2.3.1. Analysis of oxylipin and free fatty acid levels

We used ultra-high performance liquid chromatography (Agilent 1290 Infinity, Agilent Technologies, CA, USA) equipped with tandem mass spectrometry (Triple Quad 5500 system, AB SCIEX, MA, USA) [18, 19] to screen oxylipins and FFA. Briefly, a 6 mm disc of blood was obtained from the dried blood PUFAcoat® card and placed into a 96-well plate with extraction solvent (150 μL of 80% aqueous methanol) containing internal standards (0.1 ng/μL of d5-DHA and d8-AA; 0.05 ng/μL d5-EPA; 0.7 ng/μL of d5-ALA; 1 ng/μL of d4-LA; 0.01 ng/μL of d8-12S- hydroxy-eicosatetraenoic acid (HETE), d4- leukotriene B4 (LTB4) and d4-13S- hydroxy-octadecadienoic acid (HODE)). The plate was covered and gently shaken on a plate shaker for 30 min at room temperature. The extraction from each well was transferred to a fresh well in a new plate, sealed and placed in the ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) system. Analytes were captured using multiple reaction monitoring conditions of target parent and product ion as previously described [18, 19].

2.3.2. Total fatty acid analysis

Total fatty acids in the blood and breast milk were measured using dried blood and milk spot methods [17, 20]. Briefly, the dried blood/milk spot was added to 2 mL of 1% (v/v) H_2SO_4 (18 M AR grade, BDH, Sussex, UK) in anhydrous methanol (Merck, Darmstadt, Germany) in a 6 mL scintillation vial (Wheaton, Millville, USA), and heated at 70 °C for 3 h. The resultant fatty acid methyl esters (FAME) were extracted into heptanes (Merck, Darmstadt, Germany). FAME were separated and quantified using a gas chromatograph (Hewlett-Packard 6890; Palo Alto, CA, USA) equipped with a BPX70 capillary column 30 m x 0.25 mm, film thickness 0.25 μm (Trajan Scientific and Medical Pty Ltd., Victoria, Australia), programmed temperature vaporisation injector and a flame ionisation detector (FID). The injector temperature was set at 250 °C and the FID temperature at 300 °C. A programmed temperature ramp (140–240 °C) was used. Helium gas was used as a carrier at a flow rate of 1 mL/min in the column, and the inlet split ratio was set at 20:1. The identification and quantification of FAME were achieved by comparing the retention times and peak area values of unknown samples to those of commercial lipid standards (GLC-463, Nu-Chek Prep Inc., Elysian, MN, USA) using the Agilent Chemstation data system.

2.4. Nutritional management and data collection

The nutritional management approach for each unit is presented in **Supplementary Table 2**. Both centres aimed to commence total parenteral nutrition (glucose, amino acids, electrolytes and trace elements) within the first 24 h. The parenteral lipid emulsion used at the WCH was 20% SMOFlipid® containing 30% soybean oil, 30% medium-chain triglycerides, 25% olive and 15% fish oil in water consisting of LA, AA,

ALA, EPA and DHA of 37.2, 1, 4.7, 4.7 and 4.4% (by wgt of total fatty acids) respectively [14], whereas JSH used 20% Intralipos® containing 100% soybean oil in water consisting of LA and ALA of 53 and 7% (by wgt of total fatty acids) respectively but without EPA or DHA (*pers comm. manufacturer*). At the WCH enteral feeding was initiated once expressed breast milk was available (typically day 2) and in the JSH before 24 h of age. In both institutions feeds are increased at 10–20 mL/kg/d to a total of 150–160 mL/kg/d, with parenteral nutrition decreasing as enteral intake increased; if breast milk was unavailable then a preterm formula is used (currently Aptamil Gold$^+$ Preterm, Nutricia, WCH and GP-P®, Morinaga Milk Industry Co., Ltd, Tokyo, Japan, JSH). Human milk is fortified at the WCH when tolerating an enteral intake of approximately 100 mL/kg/d and at the JSH when tolerating 80 mL/kg/d. The fortifiers in use during this study did not contain PUFA. Infant clinical and intake data were collected from medical charts.

2.5. PUFA intake calculations

The PUFA (LA, AA, ALA, EPA and DHA) content of parenteral lipid emulsions and infant formula was based on the manufacture's information. The enteral PUFA (LA, AA, ALA, EPA and DHA) intake from breast milk was determined from the PUFA profiles of collected breast milk samples (**Supplementary Table 3**) assuming a milk lipid concentration of 3.5% [21]. The average breast milk concentration of the group (WCH or JSH) was used for each specific timepoint if an individual breast milk sample was not available for the corresponding time point. The total PUFA intake was calculated for the 24 h prior to the time (day) of the blood sample collection.

2.6. Statistical analysis

Differences in maternal and infant characteristics between the groups were determined using Mann-Whitney U tests or t tests for continuous variables and Fisher's exact tests for categorical variables. Linear mixed effects models were used to estimate differences in mean oxylipin, FFA and PUFA levels in the blood between the recruiting centres across time with recruiting centre, sampling time and the interaction between centre and time included as fixed effects and infant included as a random effect. The interaction term was excluded when the global test was not statistically significant ($p \geq 0.05$) and instead comparisons were made between recruiting centres across all time points. When model residuals were not normally distributed, outcome data were log transformed and differences between centres were instead expressed as ratios of geometric means with 95% confidence intervals (CIs). In exploratory analyses, a separate multiple linear regression was performed to estimate associations of each PUFA intake (parenteral and enteral) with FFA and their related oxylipins at each time point. The following variables thought to influence oxylipin levels were included in the model: total PUFA intake at postnatal day 4, 7, 14, 28, sex, gestational age and antenatal corticosteroid therapy. Oxylipin data were log-transformed to approximate normality and estimates (beta-coefficients) were back-transformed to estimate the geometric mean ratio (GMR) with 95% CI. A p value of < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS for Windows version 25.0 software (IBM, Armonk, NY, USA) and the software package R (version 3.5.1, R Foundation for Statistical Computing).

3. Results

3.1. Characteristics of the study population

This was an opportunistic study with recruitment occurring between September 2017 and March 2018 at the WCH and May 2017 and March 2018 at the JSH. During this time fifty-one infants (36 WCH and 15 JSH) were eligible, 45 were enrolled, and 44 were included in the analysis (30 infants WCH and 14 infants JSH). One infant from the WCH was excluded from analysis due to long-chain 3-hydroxyacylCoA dehydrogenase deficiency [22].

Table 1
Maternal and infant characteristics[a].

	WCH (n = 30)	JSH (n = 14)	p-values
Maternal characteristics			
Age, median (IQR) years	30 (26–33)	36 (32–38)	<0.01
BMI, median (IQR)	26.9 (22.8–28.4)	20.0 (18.8–32.5)	0.03
Chorioamnionitis	10 (33)	5 (36)	1.00
Cesarean delivery	21 (70)	13 (93)	0.13
Received antenatal corticosteroids	26 (87)	9 (64)	0.03
Infant characteristics			
Gestational age, median (IQR) weeks	29 (28–30)	27 (25–29)	0.09
Birth weight, median (IQR) grams	1260 (930–1546)	829 (653–992)	<0.01
Ponderal index, median (IQR)	22.0 (20.6–23.8)	23.5 (20.3–27.9)	0.35
Male sex	11 (37)	5 (36)	1.00
Multiple birth (no. of infants)	13 (43)	1 (7)	0.08
Apgar score ≤7 at 5 min	12 (40)	7 (50)	0.75
Oxygen treatment at 36 weeks	7 (23)	9 (64)	0.02
Respiratory (oxygen/airway) support, median (IQR) days	6 (2–34)	63 (35–96)	<0.01
Postnatal steroids	6 (20)	8 (57)	0.03
Sepsis	4 (13)	0	0.29
Necrotizing enterocolitis	1 (3)	0	1.00
Intraventricular hemorrhage	4 (13)	2 (14)	1.00
Retinopathy of prematurity	4 (13)	9 (64)	<0.01
Death	2 (7)	0	1.00

Abbreviations: JSH, Juntendo Shizuoka Hospital, Shizuoka Prefecture, Japan; WCH, Women's and Children's Hospital, Adelaide, Australia.
[a] Data are presented as n (%) unless otherwise indicated.

Relative to the WCH cohort, mothers of infants from the JSH were older and leaner, their infants' birthweight was less and duration of respiratory support longer. More JSH infants required oxygen at 36 weeks postmenstrual age and developed retinopathy of prematurity than the WCH infants (Table 1). JSH infants commenced enteral feeding earlier than WCH infants, however the WCH infants achieved full enteral feeding almost one week earlier than the JSH infants (Table 2). Total parenteral nutrition was commenced earlier in JSH than WCH (JSH median 0.5, interquartile range (IQR) 0 - 1.3 h, WCH median 7.5, IQR 2 - 14.3 h) whereas lipid emulsion was commenced later (JSH median 92, IQR 68 - 121 h, WCH median 17, IQR 11.2 - 25.5 h); there were no differences in number of days of total parenteral nutrition or lipid emulsion between the hospitals. All infants received at least some breast milk.

3.2. PUFA intake

The PUFA intake (LA, AA, ALA, EPA and DHA) from both parenteral lipid emulsion and enteral feeding at each time point are shown in Figs. 1-5 (see **Supplementary Table 3** for breast milk PUFA profile). Total LA, AA, ALA, EPA and DHA intakes on day 4 and 7 were higher in WCH infants compared with JSH infants. There were no significant differences in PUFA intake between the groups on days 14 and 28.

3.3. Longitudinal changes in total blood PUFA, FFA and their metabolites

3.3.1. Total whole blood omega-6 LA, free LA and LA derived oxylipin

Total LA in both groups gradually increased to more than double by postnatal day 28 compared with the cord blood samples (Fig. 1**a, Supplementary Table 4**). Although total LA at day 4 in WCH infants was significantly higher compared with JSH infants, this was reversed by day 14. Free LA was also significantly higher in the WCH infants at

Table 2
Nutritional management[a].

	WCH (n = 30)	JSH (n = 14)	p-values
Age enteral feeds commenced (hrs)	23 (15–47)	9 (7–19)	<0.01
Age full enteral feeding achieved[b] (days)	9 (7–13)	15.5 (13–19)	<0.01
Received parenteral lipid emulsion, n (%)	22 (73)[c]	11 (79)[d]	1.00
Age parenteral lipid emulsion commenced (hrs)	17 (11–26)	92 (68–121)	<0.01
Duration of parenteral lipid emulsion (days)	8 (5–11)	5 (4–9)	0.14
Received total parenteral nutrition, n (%)	22 (73)	14 (100)	0.04
Age total parenteral nutrition commenced (hrs)	8 (2–14)	1 (0–1)	<0.01
Duration of total parenteral nutrition (days)	11 (6–15)	14 (10–17)	0.22

Abbreviations: JSH, Juntendo Shizuoka Hospital, Shizuoka Prefecture, Japan; WCH, Women's and Children's Hospital, Adelaide, Australia.
[a] Data are presented as median (interquartile range) unless otherwise indicated.
[b] Enteral intake of > 120 mL/kg/d and maintained for three days.
[c] All received SMOFlipid®.
[d] All received Intralipos®.

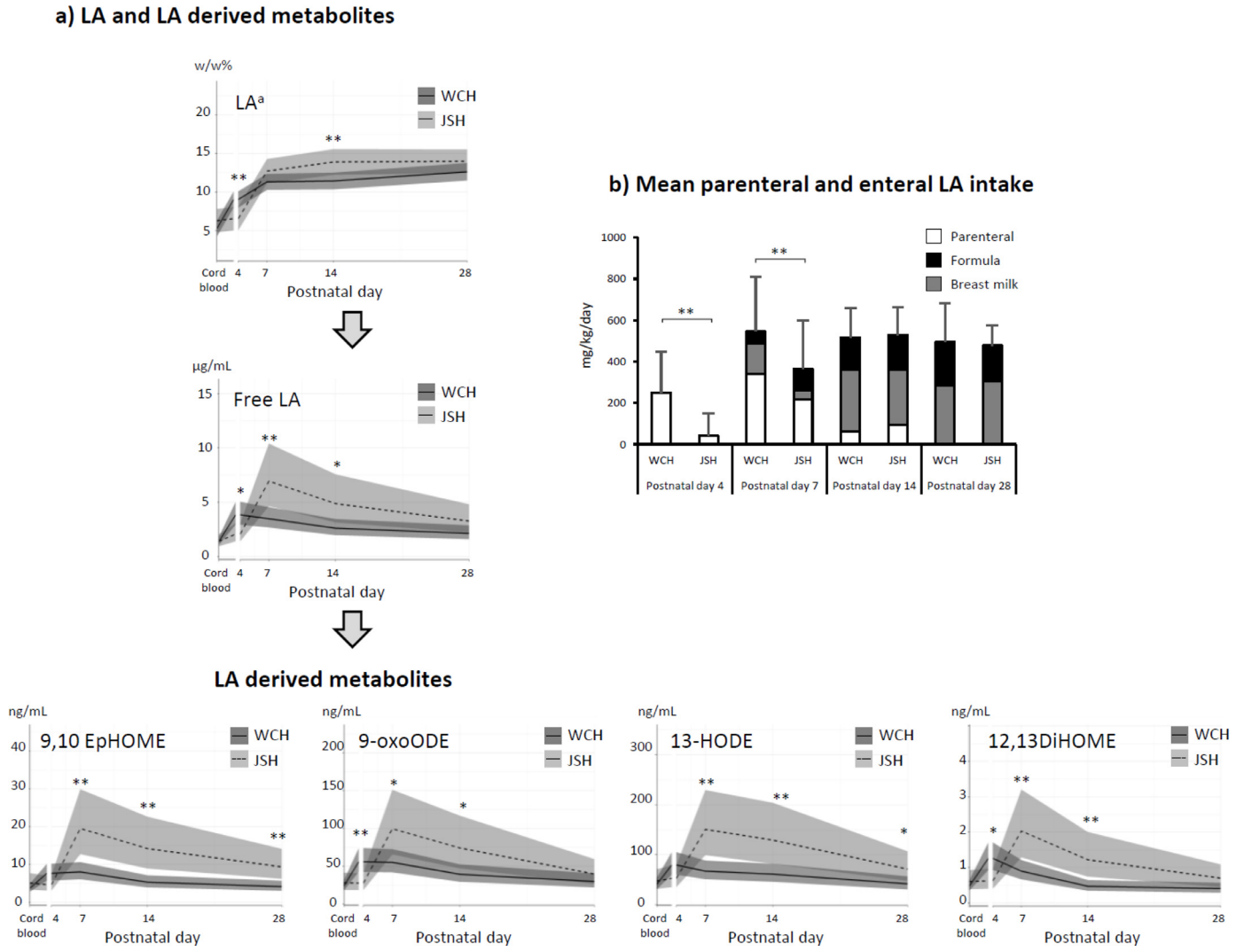

Fig. 1. n-6 LA and metabolites in preterm infants born in Australia (WCH) and Japan (JSH). (1a), Estimated geometric mean (solid line) and 95% confidence interval (shaded area) for LA, free LA and LA derived metabolites (oxylipins) in cord blood and on postnatal days 4, 7, 14 and 28. The data were log-transformed for analysis unless otherwise indicated. A statistically significant interaction effect between hospital and time was found in LA, free LA and all LA derived oxylipins, where comparisons between recruiting centres at each time point were made. (1b), Mean intake of LA (error bars represent standard deviation) on postnatal days 4, 7, 14 and 28.
[a] data were not log-transformed
* $p < 0.05$; ** $p < 0.01$, WCH vs. JSH at each time point.
LA, linoleic acid; EpHOME, epoxy-hydroxy-octadecenoic acid; oxo-ODE, oxo-octadecadienoic acid; HODE, hydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid.

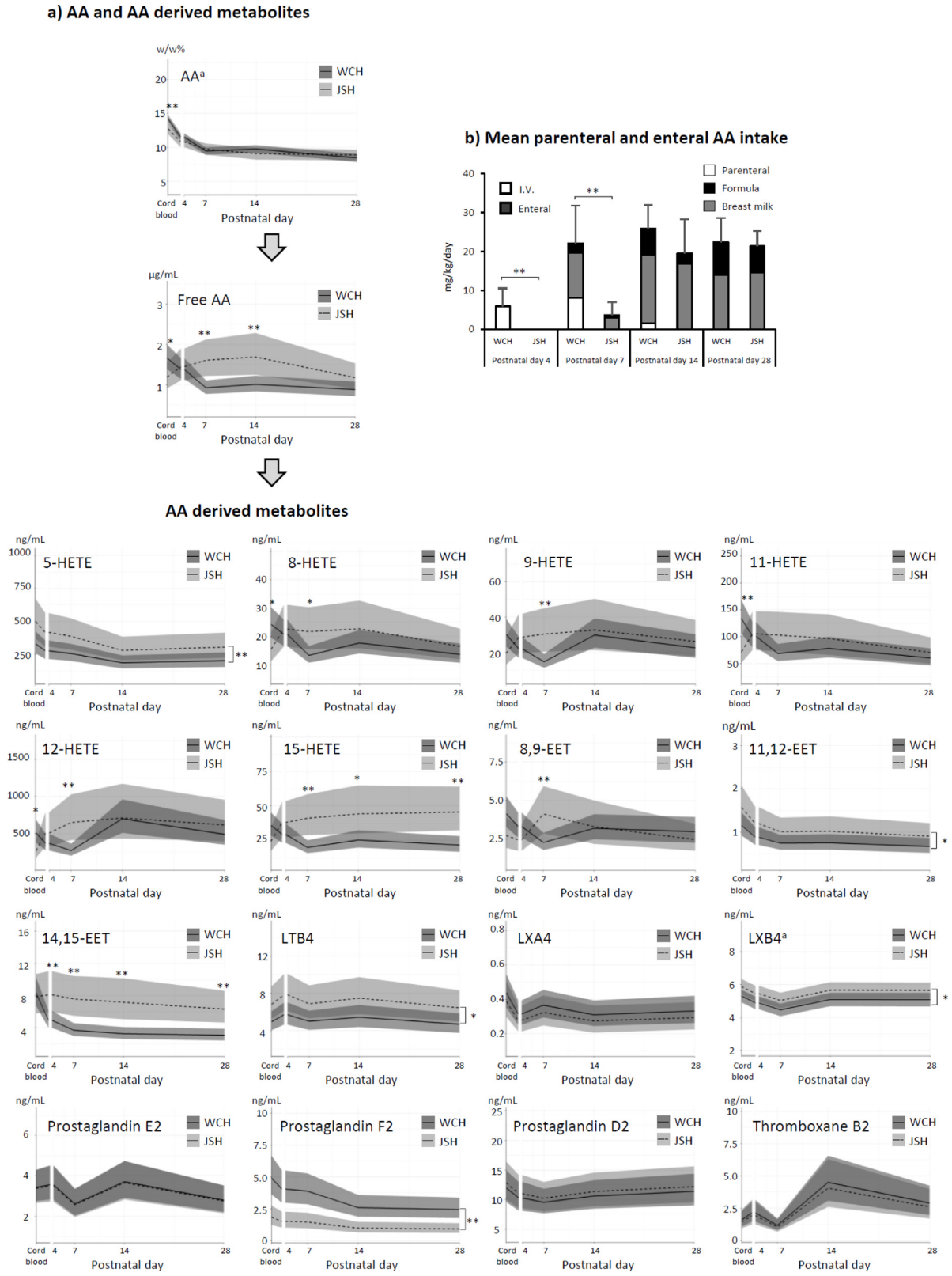

Fig. 2. n-6 AA and metabolites in preterm infants born in Australia (WCH) and Japan (JSH) (2a), Estimated geometric mean (solid line) and 95% confidence intervals (shaded area) for AA, free AA and AA derived metabolites (oxylipins) in cord blood and on postnatal days 4, 7, 14 and 28. The data were log-transformed for analysis unless otherwise indicated. A statistically significant interaction effect between hospital and time was found in AA, free AA and 7 AA derived oxylipins (8- HETE, 9-HETE, 11- HETE 12- HETE, 15- HETE, 8,9-EET, 14, 15-EET), where comparisons between recruiting centres at each time point were made. For the remaining AA derived oxylipins, an overall comparison between recruiting centres across all time points was reported (indicated on the right with statistical significance shown as described below). (2b), Mean intake of AA (error bars represent standard deviation) on postnatal days 4, 7, 14 and 28.

[a] data were not log-transformed

* $p < 0.05$; ** $p < 0.01$, WCH vs. JSH at each time point.

AA, arachidonic acid; HETE, hydroxy-eicosatetraenoic acid; EET, epoxy-eicosatrienoic acid; LTB4, leukotriene B4; LX, lipoxin.

day 4 compared with the JSH infants, which was reversed by day 7 and 14 (Fig. 1**a, Supplementary Table 5**).

Four LA-derived oxylipins (epoxy-hydroxy-octadecenoic acid (9,10 EpHOME), 9- oxo-octadecadienoic acid (oxo-ODE), 13-HODE and 12,13 dihydroxy-octadecenoic acid (DiHOME)) were detected (Fig. 1**a, Supplementary Table 6**). At day 4, 9-oxo-ODE and 12,13DiHOME were significantly higher in WCH than JSH infants. By day 7 and beyond the four LA-derived oxylipins detected were significantly higher in JSH infants compared with WCH infants (**Supplementary Table 6**).

3.3.2. Total whole blood omega-6 AA, free AA and AA derived oxylipin

Total AA was significantly higher in the cord blood of the WCH group with levels decreasing over time and no differences were observed between the groups at any time point thereafter (Fig. 2**a, Supplementary Table 3**). Cord blood free AA levels were significantly higher in WCH infants compared with JSH infants. By day 4 free AA concentration was higher in JSH infants which was maintained through day 7 and 14 (Fig. 2**a, Supplementary Table 5).** Sixteen AA- derived oxylipins were detected (Fig. 2**a, Supplementary Table 6**) with 5- and 12- HETE the most abundant. 8-, 11- and 12-HETE were significantly higher in the cord blood of WCH infants than JSH infants. At various times across days 4 to 28, six AA-derived oxylipins (8-, 9-, 12-, 15-HETE, 8,9- and 14,15- EET) were significantly higher in the JSH infants compared with the WCH infants.

The JSH infants had overall higher 5-HETE, 11,12-EET, LTB4 and LXB4 levels compared with the WCH infants (**Supplementary Table 6**), and overall lower Prostaglandin F2 levels.

3.3.3. Total whole blood omega-3 ALA, free ALA and ALA derived oxylipin

No differences in total ALA were found between the groups (Fig. 3**a, Supplementary Table 4**). Free ALA levels at day 4 were significantly higher in WCH infants (Fig. 3**a, Supplementary Table 5**). At day 4, ALA-derived oxylipin 9- hydroxy-octadecatrienoic acid (HOTrE) was significantly higher in WCH infants compared with JSH infants but by day 7 this was reversed (Fig. 3**a, Supplementary Table**6).

3.3.4. Total blood omega-3 EPA, free EPA and EPA derived oxylipin

Total EPA was higher in the cord blood of JSH infants compared with WCH infants. At day 4 through to day 14 total EPA levels were significantly higher in WCH infants (Fig. 4**a, Supplementary Table 4**). Free EPA (Fig. 4**a, Supplementary Table 5**), and the EPA derived oxylipin 18- hydroxy-eicosapentaenoic acid (HEPE), at day 4 and 7 were significantly higher in WCH infants compared with JSH infants (Fig. 4**a, Supplementary Table 6**).

3.3.5. Total blood omega-3 DHA, free DHA and DHA derived oxylipin

Total cord blood DHA was significantly higher in the JSH infants. Infant DHA levels decreased by around 30% in both groups thereafter with no significant differences between groups (Fig. 5**a, Supplementary Table 4**). Free DHA levels in the JSH infants at day 14 and 28 were significantly higher than those in the WCH infants (Fig. 5**a, Supplementary Table 5**). Five DHA derived oxylipins were detected (4-hydroxy-docosahexaenoic acid (HDHA), 7-HDHA, 14-HDHA 16,17EpDPA and 19,20EpDPA). The DHA derived oxylipin 19,20EpDPA, cord blood, at day 4 and 7 were significantly higher in the JSH infants compared with the WCH infants (Fig. 5**a, Supplementary Table 6**). Overall, 4-HDHA and 16,17EpDPA levels were significantly higher in JSH infants (Fig. 5**a, Supplementary Table 6**).

3.4. Associations between total PUFA intakes, FFA and oxylipins

On day 4 most of the measured oxylipins and their related FFA were significantly and positively associated with their parent PUFA intakes (Table 3). The exception was for AA where only one oxylipin (Prostaglandin F2) was positively associated AA intake. Significant negative associations were apparent for 5-HETE and EPA and DHA intake (Table 3); PUFA intake was predominantly parenteral at this time. Most of the measured FFA and the oxylipins 9-HOTrE, 18-HEPE and 7-HDHA were positively associated with their parent PUFA intake on day 7 (Table 3). A negative association occurred for 8-, 9-, 12-HETE and 14,15EET with EPA intake at this time. By day 14, significant positive associations were seen between EPA intake and free EPA, and 18-HEPE. 4-HDHA and 19,20EpDPA were positively associated with DHA intake on day 28; PUFA intake was predominantly enteral at this time.

4. Discussion

We have characterized whole blood PUFA levels and their derivatives (FFA and oxylipins) in preterm infants born <31 weeks' gestation during the first month of postnatal life from an Australian (WCH) and Japanese (JSH) neonatal unit. A characteristic of this study is the difference in clinical practice relating to the type of parenteral lipids used and the timing of their introduction. Japanese clinicians tend to delay initiation of parenteral lipids (commencing at ~4 days of age) and use a preparation composed of vegetable oil. In contrast Australian practice is to initiate parenteral lipids in the first day of life and use a preparation that is a mixture of MCT oil, vegetable oil and marine oil. This diversity in practice resulted in clear differences in the pattern of PUFAs and their derivatives seen in the blood of the infants.

Despite the differences in the intake of individual PUFAs, this did not always translate to differences in the percent composition of the PUFAs between the groups. This was particularly true of AA, ALA and DHA. While small differences were seen in LA levels, it was only in EPA that large differences were seen. In contrast, changes in the levels of all free PUFA concentrations were clearly visible in the blood spots. Because it is generally thought that FFA are the direct precursors of their oxygenated compounds known as oxylipins our results are highly relevant. This importance is underscored by the close relationship between the levels of many of the free PUFA and their oxylipins. This is particularly true for free LA and the 4 oxylipin derivatives known as oxidized linoleic acid metabolites (OXLAMs) as well as free ALA and free EPA and their related oxylipins. Some free PUFA levels did not have a close relationship with the oxylipins we measured including free AA where only seven of the 16 oxylipins measured followed similar patterns to the free AA levels, and free DHA where only one of five oxylipins seemed to be directly related to free DHA levels.

The changes in LA, ALA and EPA blood concentrations, and their oxylipin metabolites, were primarily driven by the fatty acid composition of the parenteral lipid used. LA and ALA were present in both parenteral lipids used (SMOFlipid® WCH infants, Intralipos® JSH infants), which is why their concentrations peaked in blood upon administration, as did concentrations of their oxylipin metabolites. SMOFlipid® contained EPA, consistent with the rise in blood EPA and its metabolites in the WCH infants.

Although DHA was present in SMOFlipid® (WCH infants), total DHA percent composition did not differ significantly between the groups over time. Tissue reserves of DHA are likely higher in Japanese than Australian infants due to higher maternal intake of DHA by Japanese women [23]. Hence, supplemental DHA is likely to partition into tissues of WCH infants, where reserves of DHA and its metabolites are expected to be lower than Japanese preterm infants.

AA percent composition did not differ over time between the groups, but free AA and its metabolites (with the exception of prostaglandin F2) were lower in preterm infants who received SMOFlipid® (WCH infants) compared with infants receiving Intralipos® (JSH infants), despite higher intakes of AA in the former, with negative associations between some AA derived oxylipins and n-3 PUFA intakes. This is likely due to displacement of AA from blood by EPA and DHA present

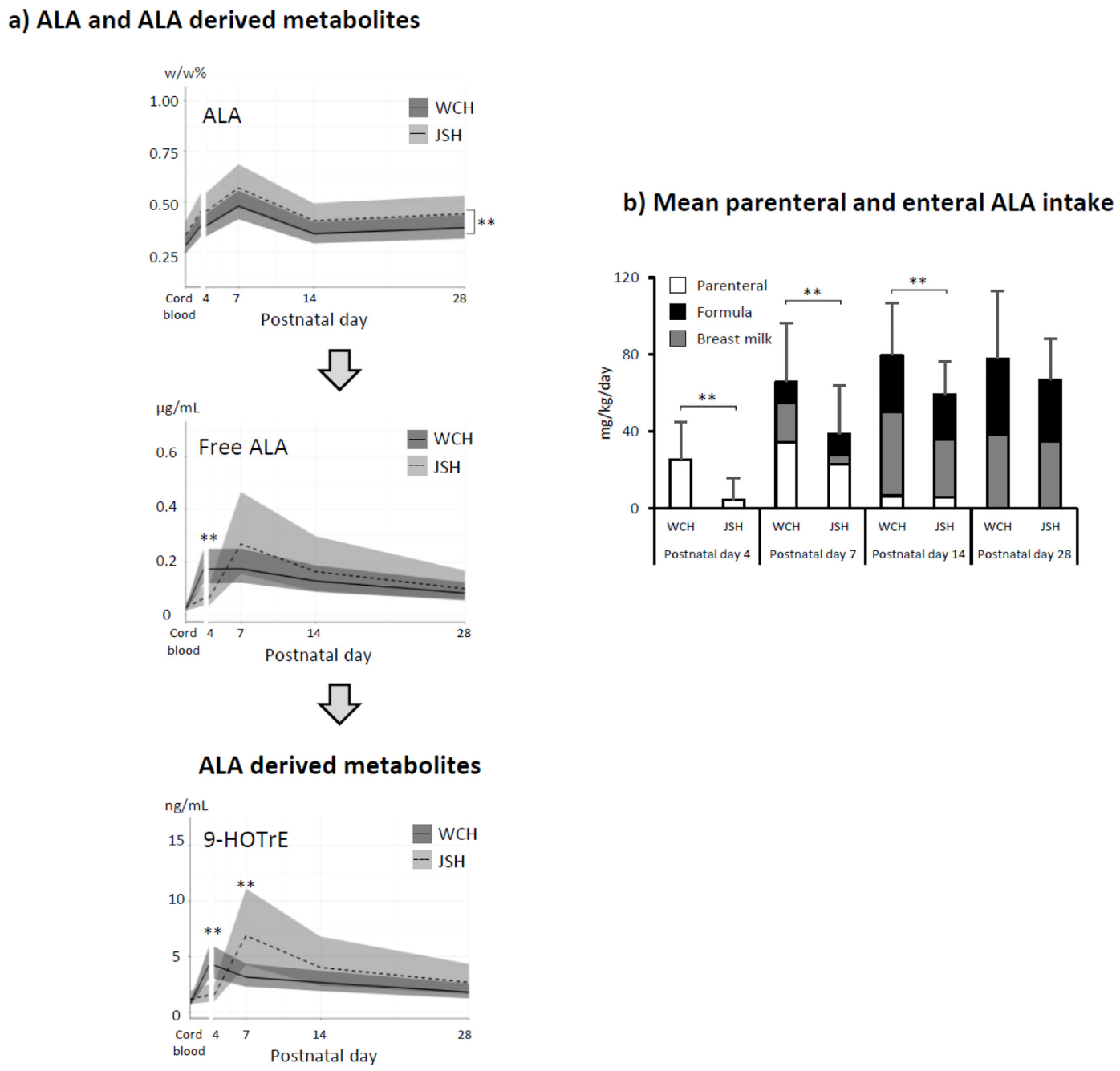

Fig. 3. n-3 ALA and metabolites in preterm infants born in Australia (WCH) and Japan (JSH) (3a), Estimated geometric mean (solid line) and 95% confidence intervals (shaded area) for LA, free LA and LA derived metabolites (oxylipins) in cord blood and on postnatal days 4, 7, 14 and 28. The data were log-transformed for analysis unless otherwise indicated. A statistically significant interaction effect between hospital and time was found in free ALA and ALA derived oxylipins, where comparisons between recruiting centres at each time point were made. For ALA, an overall comparison between recruiting centres across all time points was reported (indicated on the right with statistical significance shown as described below). (3b), Mean intake of ALA (error bars represent standard deviation) on postnatal days 4, 7, 14 and 28.

* $p < 0.05$; ** $p < 0.01$, WCH vs. JSH at each time point.

ALA, α-linolenic acid; HOTrE, hydroxy-octadecatrienoic acid.

in SMOFlipid®. Reduced levels of pro-inflammatory oxylipins derived from n-6 PUFAs associated with n-3 PUFA supplementation in an animal model has been described [32], suggesting that n-3 PUFAs may reduce the release of esterified AA or the synthesis of AA-derived oxylipins

A unique feature of this study was that the blood samples were collected on filter paper (PUFAcoat® card) as dried blood spots (DBS). This allowed for the easy collection, transport and storage of samples in this international study. We have previously reported that our DBS stabilises the fatty acids in blood for long periods [17] and more recently have reported the utility of our DBS system for the measurement of free PUFA [18] and their oxylipins [19]. The current study demonstrates the sensitivity of the DBS system with most free PUFA and many oxylipins responding to dietary PUFA intake in predictable ways. Whether the fact that some free PUFA and some oxylipins responded to dietary PUFA intake while some did not may be due to limitations in the DBS system and remains to be explored.

Recently, concern has been expressed that high dietary LA intake

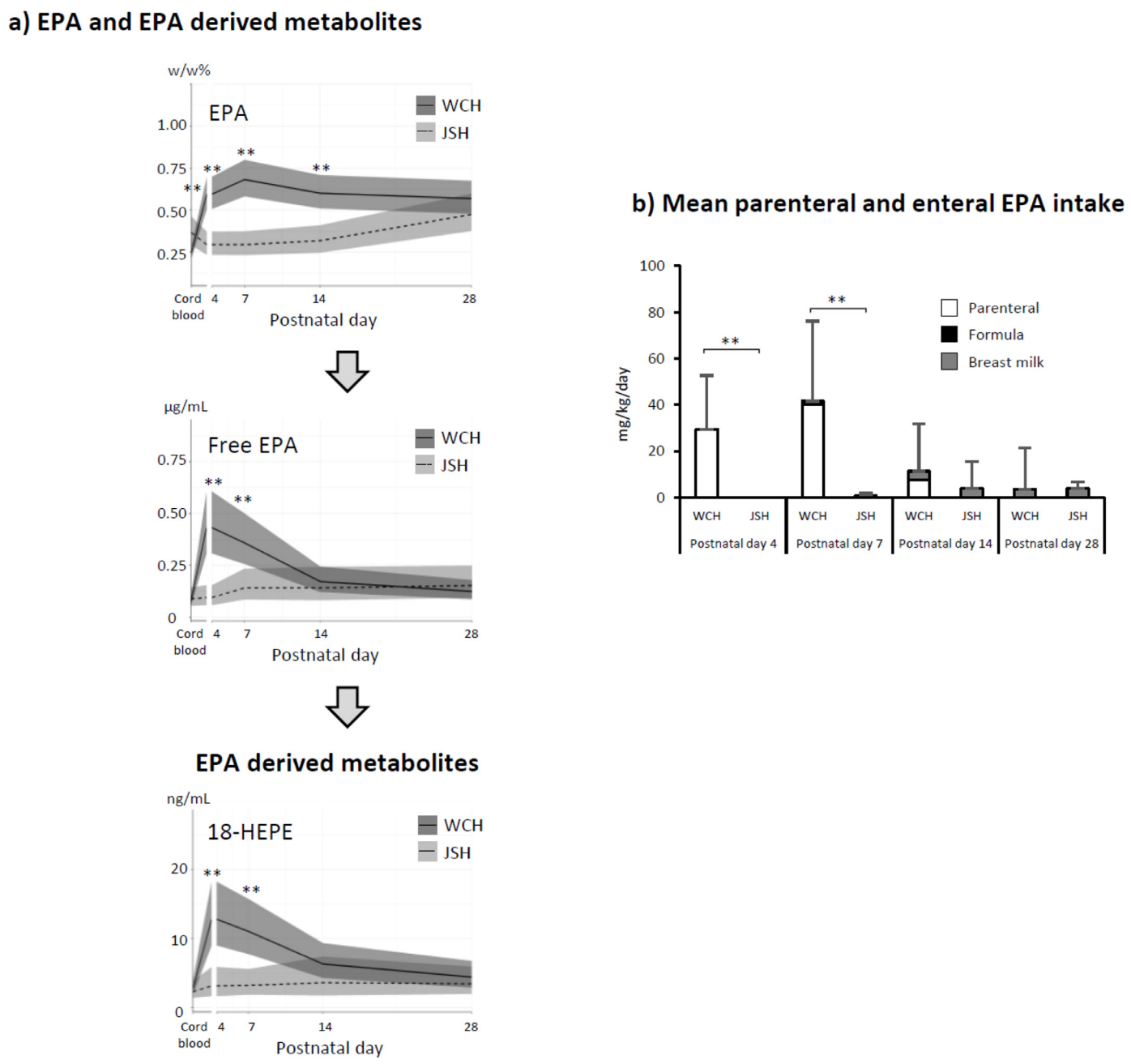

Fig. 4. n-3 EPA and metabolites in preterm infants born in Australia (WCH) and Japan (JSH) (4a.), Estimated geometric mean (solid line) and 95% confidence intervals (shaded area) for EPA, free EPA and EPA derived metabolites (oxylipins) in cord blood and on postnatal days 4, 7, 14 and 28. The data were log-transformed for analysis unless. A statistically significant interaction effect between hospital and time was found in EPA, free EPA and EPA derived oxylipins, where comparisons between recruiting centres at each time point were made. (4b), Mean intake of EPA (error bars represent standard deviation) on postnatal days 4, 7, 14 and 28. * $p < 0.05$; ** $p < 0.01$, WCH vs. JSH at each time point.
EPA, eicosapentaenoic acid; HEPE, hydroxy-eicosapentaenoic acid.

leads to an increase in levels of the bioactive LA derived oxylipins, which have been implicated in the pathogenesis of some chronic diseases in adults [23]. In our study, LA derived oxylipins including 9,10EpHOME, 9-oxo-ODE, 13-HODE and 12,13DiHOME were detected. The differing proportions of PUFA in currently available lipid emulsions contributes to the blood PUFA profile in preterm infants [24, 25]. In a systematic review of parenteral lipid emulsion in clinical use showed no differences with respect to important clinical outcomes [26]. However, LA derived oxylipin levels in preterm infants who received lipid emulsion and the associations between the LA derived oxylipins and LA intake from lipid emulsion have not been investigated at all. The present study indicates that LA intake from lipid emulsion results in an increase in levels of LA derived oxylipins that may have inflammatory activity. Interestingly, peak levels of LA derived oxylipins in the Japanese infants were more than twice those seen in the Australian infants, though this was not explained by the intake of LA from lipid emulsion. In our previous study, Intralipid was shown to contain higher amounts of LA derived oxylipins compared to SMOFlipid® [27]. It is therefore likely that differences in the peak levels of LA derived oxylipins between the groups are due to the LA derived oxylipins that are administered with the lipid emulsions.

The omega-3 PUFA-derived oxylipins have been shown to lead to anti-inflammatory effect in animal models [28]. The EPA derived oxylipin, 18-HEPE, is the precursor of the anti-inflammatory E-resolvin

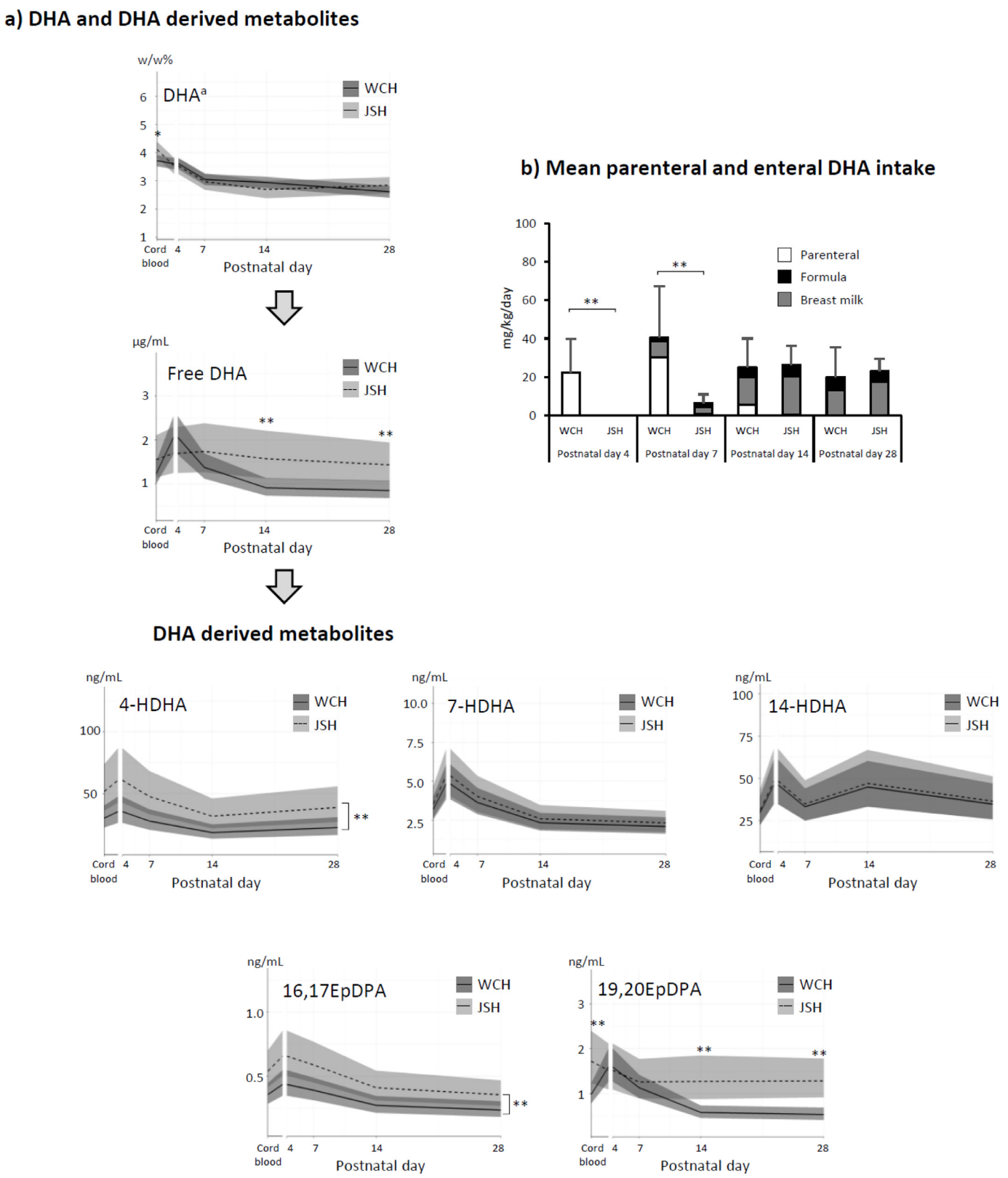

Fig. 5. n-3 DHA and metabolites in preterm infants born in Australia (WCH) and Japan (JSH) (5a), Estimated geometric mean (solid line) and 95% confidence intervals (shaded area) for DHA, free DHA and DHA derived metabolites (oxylipins) in cord blood and on postnatal days 4, 7, 14 and 28. The data were log-transformed for analysis unless otherwise indicated. A statistically significant interaction effect between hospital and time was found in DHA, free DHA and 1 DHA derived oxylipins (19,20-EpDPA), where comparisons between recruiting centres at each time point were made. For the remaining DHA derived oxylipins, an overall comparison between recruiting centres across all time points was reported (indicated on the right with statistical significance shown as described below). (5b), Mean intake of DHA (error bars represent standard deviation) on postnatal days 4, 7, 14 and 28.

[a] data was not log-transformed

* $p < 0.05$; ** $p < 0.01$, WCH vs. JSH at each time point.

DHA, docosahexaenoic acid; HDHA, hydroxy-docosahexaenoic acid; EpDPA, epoxy Docosapentaenoic Acid.

Table 3
Associations between total PUFA intakes, FFA and oxylipin levels at each time point.

	Dependent variables	Predictor variables	exp(β)[a]	(95% CI)	Adjusted R^{2}[b]	*p*
Day 4	Free LA	LA intake	1.003	(1.001–1.004)	0.42	<0.01
	9,10 EpHOME	LA intake	1.003	(1.001–1.004)	0.35	<0.01
	9-Oxoode	LA intake	1.003	(1.001–1.004)	0.35	<0.01
	13-HODE	LA intake	1.002	(1.001–1.004)	0.29	<0.01
	12,13DiHOME	LA intake	1.003	(1.001–1.005)	0.35	<0.01
	5-HETE	EPA intake	0.989	(0.978–0.997)	0.14	0.04
	5-HETE	DHA intake	0.991	(0.983–0.997)	0.14	0.04
	Prostaglandin F2	AA intake	1.201	(1.111–1.297)	0.42	<0.01
	Free ALA	ALA intake	1.040	(1.023–1.057)	0.42	<0.01
	9-HOTrE	ALA intake	1.022	(1.008–1.037)	0.27	<0.01
	Free EPA	EPA intake	1.050	(1.036–1.065)	0.62	<0.01
	18-HEPE	EPA intake	1.047	(1.031–1.063)	0.64	<0.01
	Free DHA	DHA intake	1.008	(1.002–1.013)	0.43	<0.01
	7-HDHA	DHA intake	1.010	(1.003–1.017)	0.45	<0.01
	14-HDHA	DHA intake	1.012	(1.002–1.022)	0.34	0.02
	16,17EpDPA	DHA intake	1.012	(1.004–1.021)	0.30	<0.01
	19,20EpDPA	DHA intake	1.008	(1.002–1.015)	0.36	0.01
Day 7	Free LA	LA intake	1.001	(1.000–1.002)	0.27	0.01
	9,10 EpHOME	LA intake	1.001	(1.000–1.002)	0.26	0.03
	9-Oxoode	LA intake	1.001	(1.000–1.002)	0.20	<0.01
	13-HODE	LA intake	1.001	(1.000–1.002)	0.30	<0.01
	12,13DiHOME	LA intake	1.001	(1.000–1.002)	0.19	0.02
	8-HETE	EPA intake	0.995	(0.991–1.000)	0.20	0.03
	9-HETE	EPA intake	0.994	(0.989–1.000)	0.25	0.04
	12-HETE	EPA intake	0.992	(0.986–0.999)	0.20	0.03
	12-HETE	DHA intake	0.995	(0.990–1.000)	0.20	0.04
	14,15EET	EPA intake	0.993	(0.987–0.999)	0.14	0.03
	Free ALA	ALA intake	1.011	(1.004–1.018)	0.25	<0.01
	9-HOTrE	ALA intake	1.009	(1.002–1.015)	0.23	<0.01
	Free EPA	EPA intake	1.018	(1.014–1.021)	0.76	<0.01
	18-HEPE	EPA intake	1.026	(1.019–1.033)	0.57	<0.01
	7-HDHA	DHA intake	1.005	(1.001–1.009)	0.28	0.01
Day 14	Free EPA	EPA intake	1.028	(1.013–1.044)	0.39	<0.01
	18-HEPE	EPA intake	1.025	(1.005–1.044)	0.24	0.01
Day 28	4-HDHA	DHA intake	1.040	(1.006–1.076)	0.23	0.02
	19,20EpDPA	DHA intake	1.028	(1.005–1.051)	0.33	0.02

Note: Data only shows the statistically significant associations between oxylipin as dependent variables and each PUFA intake as predictor variables on each time point.

Abbreviations: CI, confidential interval; LA, linoleic acid; AA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; EpHOME, epoxy-hydroxy-octadecenoic acid; oxo-ODE, oxo-octadecadienoic acid; HODE, hydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; HETE, hydroxy-eicosatetraenoic acid; EET, epoxy-eicosatrienoic acid; HOTrE, hydroxy-octadecatrienoic acid; HEPE, hydroxy-eicosapentaenoic acid; HDHA, hydroxy-docosahexaenoic acid; EpDPA, epoxy docosapentaenoic Acid.

[a] exp(β) shown as changes in the ratio of estimated geometric means of the dependent variable.

[b] Adjusted for sex, gestational age and antenatal corticosteroid therapy.

family, which inhibits neutrophil infiltration [29] and reduces lipopolysaccharide-triggered TNF-α formation [30]. Furthermore, the DHA derived oxylipins including HDHA and EpDPA are the precursors of the resolvin D and protectin D families, which have been shown to have anti-inflammatory effects in a variety of models [4]. Previous studies demonstrated that n-3 PUFA supplementation containing high DHA and EPA led to a dose-dependent increase in their derived oxylipins, with an increase of EPA and DHA of blood cell membrane [31]. In an animal model, dietary n-3 PUFA supplementation promoted the synthesis of anti-inflammatory oxylipins derived from DHA and EPA in the brain [32]. Consistent with these studies, we found that the intakes of DHA and of EPA were positively related to their respective oxylipins.

There have been limited reports of the relationship between preterm infant inflammatory conditions and oxylipins. In preterm infants n-6 PUFA derived oxylipins were not associated with the development of bronchopulmonary dysplasia [33]. In a neonatal animal model, downstream DHA and AA derivatives were shown to have the potential to improve hyperoxia-induced lung injury, speculating that decreases in DHA and AA in preterm infants after birth leads to a decrease of DHA and AA derived metabolites potentially contributing to the development of lung disease in this population [11]. Furthermore, high levels of oxylipins have been detected in breast milk which may affect the immune regulation of the gastrointestinal tract in the newborn indicating the advantage of human milk [12]. Our current study reports longitudinal oxylipin levels during the first 28 days of life, with dramatic changes of LA derived oxylipins from day 4 associated with the commencement of lipid emulsion.

Oxylipin profiles depend on the type of dietary PUFA and the type of enzymes including lipoxygenase, cyclooxygenase and cytochrome P450 [4]. However, little is known about the association between oxylipin levels in infants and enteral feeding. In our study enteral DHA and EPA intake were associated with their oxylipin levels in the blood, while LA and AA were not. The present study also shows that DHA in breast milk of Japanese women was approximately two times higher than that of Australian women. The percentages of n-3 PUFA to total fatty acids in breast milk, especially DHA, are different in each country [15, 34]. It is likely that those differences affect their related oxylipins in preterm infants.

Our study has limitations. Infant characteristics including clinical treatment, nutrition protocol, disease severity and maternal characteristics were different between the two groups, which may have contributed to the observed difference in the oxylipin levels. However, the findings may reveal that infant environment in each country can result in the differences in oxylipin levels, which may affect the clinical outcomes in preterm infants in each country. As an opportunistic study the sample size was small which prevented exploration of associations

between clinical outcomes and oxylipin levels.

In conclusion, we demonstrated the differences in the oxylipin levels between preterm infants born in Australia and Japan. These differences were likely a reflection of the PUFA concentration in the parenterally administered lipid emulsion and breast milk. Further studies to investigate the association between the oxylipin levels and nutrition and to determine whether oxylipins influence clinical outcomes in preterm infants are warranted.

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Author contributions

Suganuma, Collins, Andersen, McPhee and Gibson contributed to the study design; Suganuma, Ikeda, Ohkawa performed data collection; Liu carried out laboratory analysis; Leemaqz and Suganuma analysed the data; Suganuma, Collins, Andersen, McPhee and Gibson drafted the manuscript; All authors participated in the revision of the manuscript and approved the final manuscript.

Declaration of Competing Interest

RAG served on the Fonterra Scientific Advisory Board (to September 2018), honorarium was paid to support travel and consulting time. In addition, RAG has a patent 'Stabilising and analysing Fatty Acids in a biological sample stored on solid media' licensed to Adelaide Research and Innovation, University of Adelaide. Other authors have no conflicts of interest to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.plefa.2019.102026.

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